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Pharmacokinetic aspects of reduced nicotinamide adenin dinucleotide (NADH) in rats

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1. ABSTRACT

Nicotinamide adenine dinucleotide (NADH) plays a major role in cellular metabolism and mitochondrial dysfunction and is thought that NAD^+/NADH decrease neuronal degeneration and improve behavioral deficits. This potential use of NAD^+ or NADH as neuroprotective drugs requires an insight on the pharmacokinetic properties of these compounds. For this reason, we assessed the absorption of NADH in the small intestine *in vitro* using the everted gut sac technique. We show an enteral absorption of the intact NADH molecule. In the gut sac, NADH had a concentration-independent absorption rate of about 5% and the *in vivo* laser-induced fluorescence spectroscopy revealed a relatively quick absorption of NADH starting after a few minute reaching a plateau (about 5%) after 20-30 minutes. Theses results show that, should NADH be protected against the acidic conditions of the stomach, NADH is absorbed principally in the small intestine.

2. INTRODUCTION

Nicotinamide adenine dinucleotide in its oxidized form, NAD^+, and in its reduced form, NADH, as well as the phosphorylated forms hold a key role in cellular metabolism reactions in almost all metabolic pathways. The majority of the energy conserved during catabolism reactions occurs in the respiratory chain. Intermediates of metabolic reactions react with the coenzyme NAD^+ inside of mitochondria. The resulting NADH interacts with the NADH reductase, generating NAD^+ once more. Again, NAD^+ reacts with organic metabolites, i.e. a cycling effect starts. These reactions of inter-conversion of NADH/NAD^+ in the respiratory chain are not destructive. Normal mitochondrial function is a critical factor in maintaining homeostasis in various organs, including the brain. Mitochondrial dysfunction may cause or is involved in many pathological states like common neurodegenerative diseases (1, 2). Consequently, *in vitro* and *in vivo* measurement of NADH fluorescence is a well established

method to evaluate mitochondrial function in the brain (for review see (3, 4).

However, more recent research has revealed that NAD^+ is linked intrinsically to signaling reactions inside and outside of cells that control gene expression, Ca^{2+} mobilization, cell death and aging (5-8). Axonopathies, including axonal dysfunction are critical events in distinct degenerative processes, including Alzheimer's and Parkinson's diseases and multiple sclerosis, as well. It is thought that a raised NAD^+ activity increases the activity of NAD^+-dependent deacetylases (sirtuins) which then induce a decrease in axonal degeneration of injured neuronal cells (9).

Already in the 90s it could been shown that NADH may rescue pheochromocytoma cells from apoptosis, and more recently a NAD^+ mediated protection against neurodegeneration could be demonstrated in mice (10). It has been concluded, that therapies that protect neuronal NAD^+ might be useful in neurodegenerative disorders. Regarding aging in neuronal models there are indications that protective effects of NAD^+ synthesis are also mediated via sirtuins (for review see 5). Investigations to identify the targets and mechanisms of action of NAD^+ in mitochondria are needed. At present the dualism of $NADH/NAD^+$ in mitochondrial functions both in energy supply and in cell protection is a central area for development of ideas for nutritional and therapeutic use of these compounds. Further on, several studies have also shown that by increasing the NAD^+ and NADH levels behavioral deficits or disturbances can be alleviated. Thus, effects of NADH have been studied not only in cell cultures or brain slices, but also in animal models of brain diseases and injuries as well as in clinical trials and studies in humans.

First notice of NADH as a behaviorally active drug was made in laboratory rats in 1985, when jumping behavior was induced by NADH microinjection in cortex or substantia nigra and compared with the jumping behavior induced by amphetamine (11). On the other hand, amphetamine- induced jumping is accompanied by increases in NADH concentration in the rat brain (11). Already in the late 80ies the clinical effect of a NADH infusion in 115 Parkinson patients has been studied showing an improvement of the disability score by more than 10% in 97 patients (12). The study was initiated since a H4biopterin deficiency in the brain of Parkinson patients had been observed. It was assumed that NADH as the co-enzyme of dihydropterin reductase may yield a higher tyrosine hydroxylase activity. This hypothesis was later proved in rat pheochromocytoma cells, showing a stimulation of dopamine biosynthesis by NADH (13). Later on in an open clinical trial orally applied NADH was shown to improve the disabilities of patients with Parkinson's disease in a comparable manner as parenterally administered NADH (14, 15).

In the following years several trials assessing the therapeutic effects of NADH in patients suffering from various psychiatric diseases and syndromes have been performed,: e.g. chronic fatigue syndrome, depression, and Alzheimer's disease (16-18). Results of animal studies suggested beneficial effects of NADH in models of brain injury following transient focal ischemia in rats (19), experimental autoimmune encephalomyelitis in mice (10) as well as mnemotropic effects in old but not young Wistar rats (20). NADH, given systemically, also induced antidepressant-like effects in the Porsolt-Test in rats (21). This rapidly growing body of evidence suggests that NAD^+ and NADH may be used as novel drugs for treating neurological and psychiatric diseases (22, 23). However, this idea was contradicted by showing no cognition-enhancing effects of oral NADH in dementia (24). If we are going to use NAD^+ or NADH as drugs in man, it is necessary to be acquainted with the pharmacokinetic of the agents. However, there is little known about the fate of these compounds in the body after administration.

Until now it is not clear whether NADH may be absorbed in the intestinal tract and there is also no information about the time course of absorption available.

Aim of our study is an assessment of NADH absorption in the small intestine *in vitro*. For the determination of the absorption in the small intestine we used the everted gut sac technique established by Wilson and Wiseman (25). The time course of the NADH absorption was examined with high time resolution using the *in vivo* laser-induced fluorescence spectroscopy (4).

3. MATERIALS AND METHODS

3.1. Animals

Male Wistar rats (Schönwalde, Germany) 320 +/- 25 g, group housed under a 12-hr light-dark-schedule were used. All animal experiments were carried out following the principles of laboratory animal care and the German Law on Protection of Animals and were approved by the animal protection board of the state of Berlin „Landesamt für Gesundheit und Soziales Berlin".

3.2. Drugs

For the *in vitro* experiments the following substances were used: reduced nicotinamide adenine dinucleotide (NADH, GERBU GmbH, Germany), diazepam per injectionem (Ratiopharm GmbH, Germany) and vehicle (sodium bicarbonate buffer as stabilizer for NADH). All drugs were dissolved in vehicle directly prior to use. The concentrations of NADH used were obtained from behavioral experiments using NADH in rats (20, 21).

3.3. Absorption in the everted gut sac

The everted intestinal sacs were prepared as described by (26). The animals were anesthetized with isoflurane and euthanized by cervical dislocation. Within 1 min of euthanasia, the abdomen was opened by a midline incision; the intestine was dissected out and immersed in tyrode solution (NaCl 136.9 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.0 mM, $NaHCO_3$ 11.9 mM, NaH_2PO_4 0.4 mM and glucose 5.6 mM, adjusted to pH 7.0) saturated with carbogen (27).

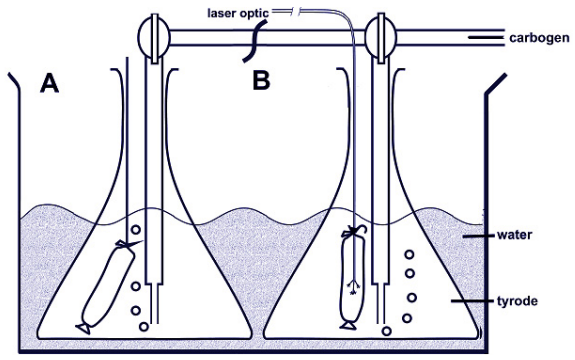

Figure 1. Schematic drawing of the experimental setup of the everted gut sac model for determination of intestinal absorption *in vitro* (A) and the combination of the everted gut sac with the laser-induced fluorescence spectroscopy for the measurement of NADH fluorescence over time (B).

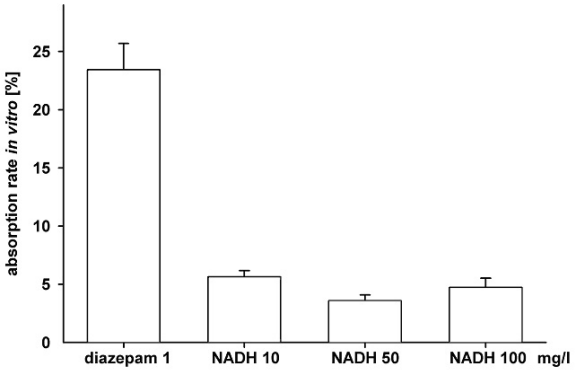

Figure 2. Absorption rate of diazepam (1mg/l) and NADH (10, 50, 100 mg/l) in the everted gut sac, an *in vitro* model of intestinal absorption after 40 minutes incubation in drug containing tyrode solution. Data are expressed as mean +/- S.E.M. (n=12-15).

The jejunum was then cleansed, divided into 3–5 cm long segments and gently everted with the mucosa showing outside and the absorption directed towards the inner lumen. The everted gut sacs were ligated on both ends and filled with incubation solution (100 micro l/cm gut length, 380 +/- 25 micro l/gut sac, 37 °C) using a cannula. Each sac was then placed in an Erlenmeyer flask (250 ml) containing 150 ml of a drug-free tyrode solution or tyrode containing either diazepam (1 mg/l) or NADH (10, 50, 100 mg/l), aerated with carbogen and kept at a temperature of 37 °C (Figure 1A). To avoid artifacts induced by decomposition of the immersed gut sac the experiment was stopped after 40 minutes incubation. The everted sacs were removed from the flask, rinsed carefully with tyrode solution, gently blotted dry, cut open and the serosal fluid transferred into small Eppendorf tubes. The sac was weighed before and after removing the serosal fluid inside of the sac and the volume of this fluid was measured.

NADH and diazepam concentrations, respectively in the tyrode outside of the gut sac in the Erlenmeyer flask were determined before and directly after incubation. Drug concentrations in the serosal fluid inside of the gut sac were determined by HPLC immediately after transfer to the Eppendorf tubes and subsequent filtration of the fluid.

3.3.1. Measurement of drug concentrations by HPLC

The concentrations of diazepam in the tyrode outside the sac and the serosal fluid inside the sac were measured by HPLC with UV-detection (extinction of 232 nm) using a 20 cm Inertsil ODS-2-Column (5 micro m, ∅ 4 mm, with a 1 cm precolumn diameter 2 mm, VDS Optilab, Germany) at a flow of 0.25 ml/min. The mobile phase consisted of sodium phosphate 20 mM (pH = 4.6), acetonitrile and isopropanol (volume ratio: 680/170/150 ml) at a temperature of 40°C.

NADH concentrations were measured by HPLC with fluorescence-detection (extinction of 340 nm, emission of 465 nm) using a 12.5 cm Spherisorb ODS-2-Column (5 µm, diameter 4.6 mm, VDS Optilab, Germany) at a flow of 1.0 ml/min. The mobile phase consisted of tetrabutylammonium hydrogensulfate 4mM dissolved in ammonium acetate 1M and methanol (volume ratio: 940/60 ml; pH = 6.3) at a temperature of 22°C. For the analysis of the chromatograms a data system CSW (DATA Apex, SR) was used. The concentrations are indicated in mg/l (Figure 2).

3.4. Measurement of the time course of NADH absorption by laser-induced fluorescence

To assess the time course of the NADH absorption in the small intestine we used the everted gut sac technique combined with the laser-induced fluorescence spectroscopy (4). The gut sac was prepared as described above; additionally the glass fiber of the laser optic was inserted through one end of the gut sac along the centre-line and secured with a ligature. Afterwards the gut sac was filled with tyrode solution. Following insertion into the Erlenmeyer flask the glass fibre was positioned in the middle of the gut sac not directed towards the gut wall, so collection of fluorescence signals stemming from the gut could be minimized (Figure 1B).

For the determination of the time course of the NADH absorption a fluorescence detector (Laser Fluoroscope 302, IOM, Berlin, Germany), coupled into the glass fiber (fused silica, diameter: 100 micro m), was used (28). The detector uses a pulsed nitrogen laser (lambda =337 nm, P=30 micro J, t=350 ps) as light source measured four times per minute. A second fiber of the same diameter, positioned directly beside the 'stimulating' glass fiber (Figure 1B), collects part of fluorescence and randomly back-scattered light to lead it back to the detection unit. Temporal and spectral gating isolates the NADH fluorescence. Measurement of NADH in an aqueous solution showed a direct linear correlation between the NADH concentration and the fluorescence intensity measured (Figure 3).

In this experiment the NADH concentration in the tyrode solution surrounding the gut was 10 mg/l. After

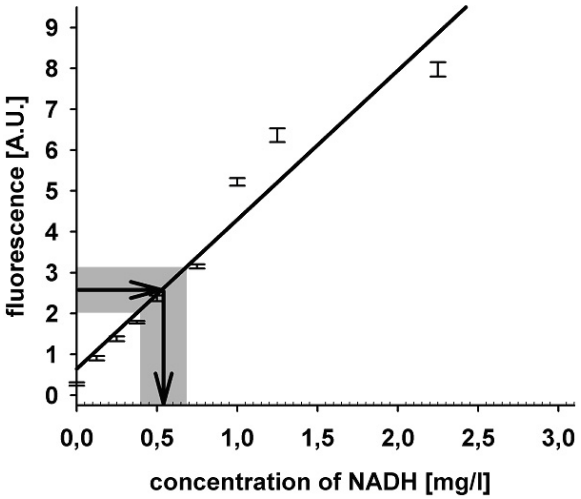

Figure 3. Calibration curve describing the concentration – fluorescence – relation of NADH in aqueous solution. The arrows designate the mean fluorescence intensity in the inner lumen of the gut sac after 40 minutes incubation of the gut sac in NADH containing tyrode solution (10mg/l), with the grey area indicating the standard errors of the mean (n=15). The data points represent the NADH fluorescence in arbitrary units and are shown as mean +/- S.E.M. from each NADH concentration determined, respectively.

40 minutes of immersion in the drug-containing buffer the measurement was stopped and gut segments were rinsed carefully at the outside. The concentrations of NADH in the serosal fluid were determined at first by HPLC with fluorescence detection followed by determination with the laser-induced fluorescence spectroscopy (n=15), after establishing a calibration curve beforehand (Figure 3) to evaluate the quantitative determination of NADH by laser-induced fluorescence.

3.5. Data handling

The drug concentrations determined in the serosal fluid were set in relation to the drug concentrations in the outer tyrode solution and the percentage of absorption calculated.

Data describing changes in NADH fluorescence over time are expressed in arbitrary units [A.U.]. The intensity of the fluorescence is dependent of the intensity of the excitation light. To exclude artifacts induced by changes in the excitation intensity by the laser energy the fluorescence measured was set in relation to the power of the laser.

3.6. Statistical analyses

The data describing the absorption of NADH and diazepam were analyzed using a one way ANOVA followed by the Holm Sidak post hoc test. The data describing the time course of the NADH absorption were analysed using a one way ANOVA on repeated measures followed by the Holm-Sidak Test, compared to controls. A difference of the means with an error probability of $p<0.05$ was considered significant. All data are presented as mean +/- standard error of the mean (S.E.M.).

4. RESULTS

4.1. Absorption in the everted gut sac

NADH and diazepam concentrations, respectively in the tyrode outside of the gut sac in the Erlenmeyer flask determined before and directly after incubation did not differ.

Following incubation of the everted gut sac for 40 minutes in tyrode solution containing diazepam (1 mg/l tyrode), the diazepam-concentration in the serosal fluid was 0.23 +/- 0.02 mg/l. The concentrations of NADH in the serosal fluid increased with rising NADH concentrations in the tyrode solution, starting with 0.56 +/- 0.05 mg/l at an external concentration of 10 mg/l and increasing to 2.79 +/- 0.26 mg/l at 50 mg/l and 4.72 +/- 0.80 mg/l at an external concentration of 100 mg/l. The absorption rate was independent from concentration and differed not between the three concentrations of NADH ($p=0.065$, $F= 3.063$, df 2, 46; Figure 2). The absorption rate of diazepam was markedly higher than the absorption rate of NADH ($p<0.001$, $F= 50.909$, df 3, 64; Figure 2).

4.2. Measurement of the time course of NADH absorption by laser-induced fluorescence

Preceding determination of NADH (0.0 - 10.0 mg/l) in an aqueous solution using laser-induced fluorescence showed a direct linear correlation between the NADH concentration and the fluorescence intensity measured (Figure 3).

Following immersion of the everted gut sac in a NADH solution of 10 mg/l for 40 minutes the concentration of NADH in the absorbate within the everted gut sac was 0.51 +/- 0.04 mg/l determined by HPLC with fluorescence detection and 0.51 +/- 0.10 mg/l determined by laser-induced fluorescence spectroscopy (Figure 3). The resulting absorption rate of 5.1 +/- 1.0% was not different to the absorption rate in the gut sac experiments without the optic probe inserted and measured by HPLC ($p=0.500$, $t= 0.683$, df 28).

During the first minutes after incubation of the everted gut sac into the NADH solution the NADH fluorescence determined by laser-induced fluorescence spectroscopy increased significantly ($p=0.001$, $F= 12.058$, df 2, 29; Figure 4) and reached a relatively stable level after approximately 25-30 minutes. In the control experiments using gut sacs immersed in NADH-free tyrode the NADH fluorescence did not increase (Figure 4).

5. DISCUSSION

Due to its relevant roles in oxidation/reduction reactions and in other biochemical reactions, like ADP-ribosylation or protein deacetylation it is speculated, that NAD^+/NADH could have a potential role in the treatment of neurodegenerative diseases, cancer, autoimmune disease and in the field of neuroprotection (22, 29). There are some

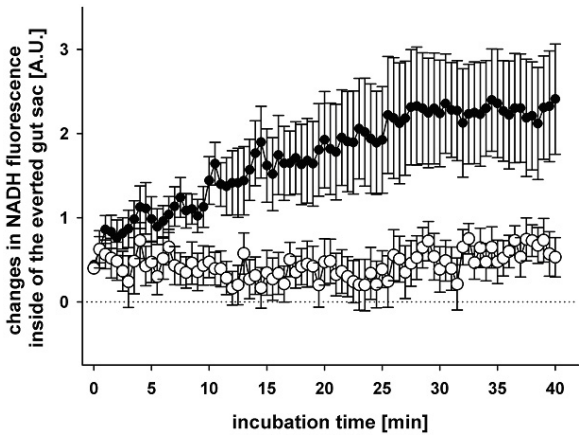

Figure 4. Change in the intensity of NADH fluorescence in the inner lumen of the everted gut sac following incubation in NADH containing tyrode solution (10mg/l;●) for 40 minutes compared to gut sacs immersed in NADH-free tyrode solution (○). Partially reproduced with permission from Wiley-VCH Verlag GmbH & Co. KGaA, (4). One way ANOVA on repeated measures followed by the Holm-Sidak Test, *p< 0.05, compared to controls (○). Data are corrected for shifts in the laser energy and expressed as means +/- SEM. (n=15).

even controversial preclinical and clinical findings assessing the therapeutic potential of NADH (24). Therefore, there is substantial interest in an extended knowledge regarding the pharmacokinetic properties of NADH. To our knowledge NADH has not been shown to be absorbed from the gastrointestinal tract. Recently, in own experiments we could show that NADH given intraperitoneally should have reached the brain, since NADH concentration was increased in the cortex shortly after administration of NADH (30), whereas oral administration had no effects on the NADH concentration in the cortex. These results suggested on one hand that NADH might be absorbed by the peritoneum and so may reach the brain. On the other hand the results indicated that oral administration was ineffective. This is confirmed by Kimura and co-workers (31) showing that oral administered NADH did not change NADH metabolite concentrations in rats. The authors suggest that NADH may be converted in the acidic milieu of the stomach or may not be absorbed in the gastrointestinal system. They could demonstrate that NADH was converted into an unknown compound after a short time of incubation under acidic conditions (31). However, whether or not NADH is absorbed in the intestinal tract remained still unknown.

Our present results demonstrate some aspects of the pharmacokinetic fate of NADH. The everted gut sac model, established more than 50 years ago (25, 26) is a reliable model for absorption screening. In comparison to other absorption models, the everted gut sac is relatively near to *in vivo* conditions but lacking some methological problems of *in vivo* perfusion models. The low volume of the absorption compartment, in relation of the absorption surface of the gut and the large external drug depot, preventing a shift in drug concentrations in the external medium during the experiments, is also an advantage of this model. The everted gut sac model has been used for quantitative information about resorption of drugs like phytopharmaceuticals (32), toxins (33) or for the assessment of co-factors affecting the bioavailability of other drugs (34).

Our results assessing the absorption in the everted gut sac amend the experiments of Kimura and co-workers (31), who showed that NADH administered orally will be decomposed in the acidic pH of the stomach. With our *in vitro* experiments using the established model of the everted gut sac we could confirm an enteral absorption of the intact NADH molecule. Under the non-acidic conditions of the small intestine absorption of NADH *in vivo* might be possible. The absorption rate of NADH in the jejunum is, according to our experiments with the everted gut sac technique, relatively low but fairly reliable. This finding is reinforced by the rather constant absorption rate of approximately 5%, independent of the concentration of NADH.

The time course of the NADH absorption *in vitro* was determined by the NADH fluorescence measurement continuously using the laser-induced fluorescence spectroscopy (4, 35). In the preceding experiment we could show that the fluorescence intensity and the concentration of NADH in an aqueous solution are closely and directly proportional and therefore even quantification of the NADH absorbed was possible. Our experiments showed a relatively quick absorption of NADH starting after few minutes and reaching a plateau after 30 minutes. The NADH determined in the inner compartment should stem from the outer medium, since incubation of everted gut sacs in a NADH-free external tyrode solution caused no changes of NADH-fluorescence in the inner compartment. Therefore NADH from the rat jejunum itself doesn't contribute significantly to the fluorescence measured.

The twofold determination of the final NADH concentration in the inner compartment revealed the accordance of the two analytical methods used. The laser-induced fluorescence, allowing repeated measurements in the same preparation, proved as a reliable method with high time resolution to observe the time course of the absorption process.

In a previous study we could show that in anaesthetized rats NADH, administered intraperitoneally and intravenously, increases the intensity of cortical NADH fluorescence. In the same study the oral administration of NADH had no effect on the cortical NADH fluorescence (30). The latter is in accordance with the findings of Kimura and co-workers (31) suggesting instability in acidic milieus. However, there are several ways to deal with the acidic environment of the stomach to achieve a pharmacological effect with drugs unstable at gastric pH. Beside special galenic formulations, like acid-proof coating of the tablets, a preferential absorption of the drug prior to passage through the stomach is possible. For example, nasal delivery of the peptide insulin has been examined for more than 30 years (36, 37). Very recently it has been

shown that intranasal NAD^+ decreases brain damage *in vivo* in a rat model of transient ischemia (19). The authors assumed that the intranasal drug delivery would help to bypass the blood-brain-barrier, as known for some peptides. In addition there are several drugs, like glyceryl trinitrate, where absorption through the mucosa in the mouth leads to a systemic action. These drugs absorbed from the mouth pass directly into systemic circulation, also bypassing the acidic pH of the stomach.

Our results prove that NADH principally can be absorbed in the small intestine, if protected against the acidic conditions during gastric passage. The laser-induced fluorescence proved to be a valuable tool for the assessment of the pharmacokinetic of NADH. In future experiments it would be of interest whether or not NAD^+/NADH, administered sublingually or using tablets coated with acid-proof coating, becomes available in the brain.

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Key Words: NADH, *In vitro*, Intestinal Absorption, Absorption Rate, Rat, Everted Gut Sac, Laser-Induced Fluorescence, HPLC

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